

Use of 16S rRNA Gene Based Clone Libraries to Assess Microbial Communities Potentially Involved in Anaerobic Methane Oxidation in a Mediterranean Cold Seep

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Abstract

This study provides data on the diversities of bacterial and archaeal communities in an active methane seep at the Kazan mud volcano in the deep Eastern Mediterranean sea. Layers of varying depths in the Kazan sediments were investigated in terms of (1) chemical parameters and (2) DNA-based microbial population structures. The latter was accomplished by analyzing the sequences of directly amplified 16S rRNA genes, resulting in the phylogenetic analysis of the prokaryotic communities. Sequences of organisms potentially associated with processes such as anaerobic methane oxidation and sulfate reduction were thus identified. Overall, the sediment layers revealed the presence of sequences of quite diverse bacterial and archaeal communities, which varied considerably with depth. Dominant types revealed in these communities are known as key organisms involved in the following processes: (1) anaerobic methane oxidation and sulfate reduction, (2) sulfide oxidation, and (3) a range of (aerobic) heterotrophic processes. In the communities in the lowest sediment layer sampled (22–34 cm), sulfate-reducing bacteria and archaea of the ANME-2 cluster (likely involved in anaerobic methane oxidation) were prevalent, whereas heterotrophic organisms abounded in the top sediment layer (0–6 cm). Communities in the middle layer (6–22 cm) contained organisms that could be linked to either of the aforementioned processes. We discuss how these phylogeny (sequence)-based findings

can support the ongoing molecular work aimed at unraveling both the functioning and the functional diversities of the communities under study.

Introduction

In the past decade, domelike structures called “mud volcanoes” have been discovered in several deep-sea sites, including the Mediterranean Sea [32]. Mud volcanoes emit large quantities of methane (an important greenhouse gas) and hydrogen sulfide, yielding marine sediment environments—also called cold seeps—that can be highly enriched in these compounds [7, 35]. For instance, the Kazan mud volcano sediment is known to be rich in methane and to contain reduced as well as oxidized forms of sulfur [54]. Life at such mud volcanoes (cold seeps) is probably largely sustained by primary producers that use methane and hydrogen sulfide as energy sources. This is reminiscent of hydrothermal vents [8, 19], where life is primarily sustained by the microbial oxidation of sulfide [3, 27, 47]. Stable carbon isotope measurements of cold seep sediments and carbonate crusts have indicated that the anaerobic oxidation of methane (AOM), rather than of sulfide, is the basis of chemoautotrophy in these deep-sea ecosystems [1, 44].

Specific methanotrophic archaeal groups, denominated ANME-1 and ANME-2, have been implicated as the main constituents of the microbial communities involved in AOM [6, 40, 49]. These organisms are either distantly (ANME-1) or closely (ANME-2) related to the (methanogenic) *Methanosarcinales* group. However, AOM by other archaea, e.g., the novel ANME-3 group,

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cannot be excluded [20, 30]. The ANME-1 and ANME-2 group archaea consume methane in a close relationship with sulfate-reducing bacteria [5, 6, 37]. To date, these organisms have not been obtained in pure culture, and hence molecular approaches are indicated to assess their diversity, prevalence, and potential function.

The occurrence of AOM in the sediments of the Kazan mud volcano has been indicated [54] by data on membrane lipids of the local microbial communities, but DNA-based studies have not yet been performed. In this work we analyze the diversity and nature of the bacterial and archaeal communities present in different Kazan sediment layers by using direct DNA-based molecular analyses. The 16S ribosomal RNA (rRNA) gene was employed as a marker for diversity. The microbial community structures found are evaluated against the chemical background of the system, in order to estimate the functional dominance of microbial AOM in the sediment layers. From this analysis, the validity of inferring ecosystem function purely from clonal phylogeny will be discussed and possible avenues toward a more sound, ecogenomics-based approach will be put forward.

Methods

Sampling. Box cores were taken from a depth of 1673 m at the summit of the Kazan mud volcano (35°25.9'N, 30°33.7'E) during the 1999 MEDINETH scientific cruise of the R/V *Professor Logachev*. Subsamples of one box core, BC19, were then taken, using sterilized aluminum corers. Based on sediment color, the core was divided in three depth layers for DNA extraction and molecular analyses: top—from 0 (surface) to 6 cm depth (brown-gray sediment, Kazan-1); middle—from 6 to 22 cm (grayish-black sediment, Kazan-2); bottom—from 22 to 34 cm (dark black to gray sediment, Kazan-3). Subsamples (1 mL) were aseptically taken from these layers by using sterile 1-mL syringes. For each layer, eight subsamples were pooled in 12-mL sterile Greiner tubes, after which these were sealed, immediately frozen at –80°C, and kept frozen upon analysis.

Chemical Analyses. Subsamples from different sediment depths were centrifuged (0°C) at $16,000 \times g$ for 10 min. The supernatants were collected with a 50-mL syringe, filtered over a 0.2- μ m membrane filter into new sterile Greiner tubes and the filtrates stored on ice. For determination of the levels of total dissolved organic carbon (TDOC), NO_3^- , NO_2^- , and NH_4^+ , SO_4^{2-} , Br^- , and Cl^- , subsamples were taken and stored at –20°C. Subsamples (2 mL) for sulfide measurements were treated with 10 μ L ultrapure 1 M NaOH per mL, and sulfide levels were determined according to standard procedures [54]; however, the sulfide determinations should be interpreted as rough estimates in the light of

the known caveats of such determinations in natural samples. Subsamples (2 mL) for PO_4^{3-} measurements were treated with 10 μ L ultrapure 1 M HCl per mL and stored at 4°C. The pelleted material from each sediment layer (10–15 g) was oven-dried (60°C, 36 h) for subsequent analyses. The NO_3^- , NO_2^- , NH_4^+ , and PO_4^{3-} concentrations were determined colorimetrically on a Nutrient Autoanalyzer 3 (Bran and Luebbe, De Meern, the Netherlands). SO_4^{2-} , Br^- , and Cl^- concentrations were determined by ion chromatography (Dionex DX-120 IC; Waters, Milford, MA, USA). TDOC was measured on a TOC analyzer (Shimadzu TOC-5050A, Shimadzu Benelux, Den Bosch, Netherlands). Water chemistry measurements were carried out in duplicate on the same water sample. The data were compared to data previously obtained by Haese and co-workers [22].

Extraction of Sediment Microbial Community DNA. The pooled samples from each depth layer were used to extract total microbial community DNA by using a modified DNA extraction protocol. Briefly, each sample was homogenized, after which 5 g (wet weight) was divided over 2-mL screw-cap tubes containing 0.5 g of a 1:1 mixture of zirconium and glass beads (diameter 0.1 mm). The mixtures were heated in a water bath at 70°C for 15 min and then put on ice. Tris buffer (0.1 M Tris, pH=9.0; 150 μ L) and lysozyme solution (50 μ L; 20 mg mL^{-1}) were added to each tube, and samples treated in a minibead beater (Biospec Products, Bartlesville, OK, USA) at 14,000 rpm for 30 s. The tubes were then horizontally shaken (1 h, 37°C, 150 rpm), after which sodium dodecyl sulfate (SDS)/proteinase K (final concentration: 0.8% SDS, 10 U mL^{-1} proteinase K) was added and the tubes were shaken (50°C, 30 min, 150 rpm) followed by centrifugation at $16,000 \times g$. Subsequently, the supernatant was collected and kept on ice. The pellet was then reextracted by the addition of 350 μ L EDTA/Tris buffer (0.5 M EDTA; 0.2 M Tris-HCl; pH 8.0) and 20 μ L SDS (25%) and heating (70°C, 5 min), and bead beating (14,000 rpm for 30 s). This procedure was repeated. After 1 h incubation at 70°C with continuous shaking (200 rpm), the samples were centrifuged (4 min, $16,000 \times g$). The EDTA/Tris extraction was repeated once more and then 400 μ L phosphate buffer (90 mM Na_2HPO_4 ; 5.5 mM NaH_2PO_4 ; pH=8.0) was added to the tubes containing the sediment/beads pellet. Samples were heated at 70°C for 5 min, treated in a minibead beater (14,000 rpm, 30 s), and incubated (30 min, 70°C) before the supernatant was collected. This procedure was repeated once. Supernatants from the same sediment section were pooled in a sterile 40-mL tube and extracted twice with an equal volume of phenol/chloroform/iso-amyl alcohol (50:49:1), and once with an equal volume of chloroform/iso-amyl alcohol (24:1). After addition of 2 vol of 100% ethanol and 0.1 vol of sodium acetate (3 M, pH=5.2) to the aqueous phase,

DNA was precipitated overnight at -20°C . The DNA pellets were washed with ice-cold 70% ethanol, air-dried, and resuspended in 1.0 mL HPLC-grade water. DNA was then cleaned by using a WIZARD DNA cleanup kit (Promega Benelux, Leiden, the Netherlands) followed by a Quick SpinTM column (Roche Nederland, Woerden, the Netherlands). Finally, DNA was concentrated to a volume of 100 μL . DNA concentrations were measured by using a Gen-equant II spectrophotometer (Pharmacia, Groningen, the Netherlands).

PCR Amplification, Cloning, and Sequencing. The 16S rRNA genes were amplified using primers specific for bacterial and archaeal 16S rRNA genes. Bacterial 16S rRNA genes were amplified by using the B8F (5'-AGAGTTTGATCMTGGCTCAG-3') forward primer [15] and the universal U1406R (5'-ACGGGCGGTG TGTRC-3') reverse primer [31]. Archaeal 16S rRNA genes were amplified with the A2F (5'-TTCCGGTTGA TCCYGCCGGA-3') forward primer [13] in combination with the universal U1406R (5'-ACGGGCGGTG TGTRC-3') reverse primer. In cases where the total amounts of polymerase chain reaction (PCR) product were low, the reaction was repeated with the same forward primer in combination with the A958-R reverse primer (5'-YCCGGCGTTGAMTCCAATT-3') [13]. PCR mixtures (25 μL) contained 10.2 mM Tris buffer, 2.3 mM MgCl_2 , 50 mM KCl, 2% DMSO, 5 μg BSA, 0.2 mM of each dNTP, 0.2 μM of each primer, and 0.5 U of *Taq* DNA polymerase. PCR was performed in a PerkinElmer

GeneAmp PCR System 9700 (PerkinElmer Applied Biosystems, Nieuwerkerk a/d IJssel, the Netherlands) using the following program: 95°C for 5 min; 35 cycles of 94°C for 1 min, 57.5°C for 30 s, 72°C for 4 min, with a final elongation step of 72°C for 7 min.

PCR products were purified using QIAquick spin columns (Invitrogen, Groningen, the Netherlands) and subsequently cloned in the pGEM-T Easy vector (Promega Benelux) using *Escherichia coli* JM109 as the host. Inserts were amplified by colony PCR, using the pGEM-T specific primers T7 (5'-TAATACGACTCACTA TAGGG-3') and SP6 (5'-GATTAGGTGACACTATAG-3'). PCR mixtures were as described above, and amplification used the following PCR conditions: 94°C for 5 min; 30 cycles of 94°C for 1 min, 48°C for 30 s, 72°C for 4 min, with a final elongation step of 72°C for 7 min.

At least 50 clones with inserts of the expected length were selected from each library and partial 16S rRNA gene sequences were determined using Big Dye version 3 cycle sequencing reactions (Applied Biosystems, Foster City, CA USA) and an ABI3100 PRISM Genetic Analyzer (Applied Biosystems). Sequencing reactions used B8F, T7, or U515 (5'-GTGCCAGCMGCCGCGG-3') forward primers for bacteria, and A2F, T7, or U515 forward primers for archaea. Partial sequences were manually edited in "Chromas 1.45" (<http://www.technelysium.com.au>) and contig assemblies were done in BioEdit (<http://www.mbio.ncsu.edu/BioEdit/bioedit.html>) according to the procedure described by Huang [24]. This yielded a majority of sequences of ~ 1000 to 1300 bp.

Table 1. Geochemistry and organic geochemistry data in three depth layers of deep-sea sediment samples, collected from an active seep at Kazan mud volcano (Eastern Mediterranean)

	Kazan-1 (0–6 cm)	Kazan-2 (6–22 cm)	Kazan-3 (22–34 cm)
Porewater chemistry data			
pH	8.02	8.69	8.73
Phosphate (μM)	0.5	0.3	0.2
Ammonium (μM)	30.0	42.7	158.5
Nitrite (μM)	1.0	0.2	0.1
Nitrate (μM)	15.7	2.0	1.0
Sulfate (mM)	31.4	32.7	3.2
Sulfide (HS^-) ^a (mM)	0	6.2	2.8
TDOC (mM)	33.4	32.9	30.3
Bromide (μM)	937.4	921.2	690.9
Chloride (mM)	626.8	612.4	377.5
Methane (mM)	0.4 ^b	2.0 ^b (~ 20) ^c	9.4 ^b (~ 60) ^c
Organic chemistry data ($\mu\text{g g dw}^{-1}$)/(% of average lipid concentration in layer) ^d			
Alkyl diethers (sulfate reducing bacteria)	0 (0%)	0.23 (4.6%)	0.21 (5.3%)
Bishomohopanol (Sulfide oxidizing bacteria)	0.19 (3.5%)	0 (0%)	0 (0%)
Diploptene/diplopterol (aerobic methane oxidizing bacteria)	0.24 (4.4%)	0.01 (0.3%)	0 (0%)
<i>sn</i> -3-Hydroxyarchaeol/archaeol/ PMI:x (AOM, methane oxidizing archaea)	0.15 (2.7%)	0.89 (17.6%)	0.40 (10.1%)

^aLimit of detection was 0.03 mM.

^bRecalculated to average concentrations over sediment layer after data from Haese *et al.* [22] and Werne *et al.* [54].

^cCalculated *in situ* concentrations according to the model presented by Haese *et al.* [22]. Without methane oxidation, the predicted methane concentration is 160 mM [22].

^dData recalculated from Werne *et al.* [53, 54].

Sequence Analyses. Chimeric sequences were detected by using the CHECK_CHIMERA utility at the Ribosomal Database Project and removed from the analyses. Nearest relatives of the remaining sequences were obtained from the Genbank database by using the basic local alignment search tool (BLAST) at the NCBI website. Sequences representing distinct phylotypes (as the criterion, 97% sequence similarity was used) and their closest relatives were aligned by using the fast aligner utility of the ARB software package [33].

Alignments were checked manually, using the secondary structure of the 16S rRNA molecule. Evolutionary distances were calculated according to the Kimura [28] two-parameter correction method, after which neighbor

joining trees were constructed with 1000 bootstrap samplings using TreeconW [52].

16S rRNA gene sequences showing 97% similarity or higher were considered to belong to the same phylotype. Related 16S rRNA gene sequences were placed within tentative taxa (between Phylum and Order) by determining the taxonomic class (using the NCBI taxonomy database) of the closest relative in GenBank of sequences that formed a phylogenetic clade. Sequences that showed no or low (below 70%) relatedness with known bacterial or archaeal phylogenetic groups were listed as unclassified.

Shannon–Weaver indices of diversity were calculated for all communities on the basis of the phylotype distribution using the PAST program (<http://folk.uio.no/ohammer/>

Table 2. Phylotype distribution and their phylogenetic grouping of sequences in three depth layers of deep-sea sediment samples, collected from an active seep at Kazan mud volcano (Eastern Mediterranean)

Domain	Phylogenetic group	Phylotype sequences		
		Kazan-1 ^a	Kazan-2	Kazan-3
Archaea	Marine Group I	1A-02(3)* 1A-06(17), 1A-09(2), 1A-10(8), 1A-15(2), 1A-36(13), 1A-41	2A-34(2), <u>2A-47*</u>	3A-15
Archaea	Novel	Not found	2A-05, 2A-08(2), 2A-09(2), 2A-11, 2A-12, <u>2A-13[#]</u> 2A-14, 2A-24, 2A-29, 2A-30, 2A-35, 2A-40(6)	3A-30, <u>3A-31[#]</u>
Archaea	Crenarchaea			
Archaea	Novel	1A-47	2A-04(3)	3A-21, 3A-27
Archaea	Halobacteriales			
Archaea	Novel	Not found	2A-01(3), <u>2A-17(13)[†]</u>	3A-04(2), 3A-05, 3A-07(15), 3A-12, <u>3A-18(3)[†]</u> 3A-33(15), 3A-35, 3A-36(9)
Archaea	Methanosarcinales			3A-10(2)
Archaea	Thermoplasmatales-related	Not found	2A-10	
Archaea	Unclassified archaea ^b	Not found	2A-26, 2A-28(3)	Not found
Bacteria	Acidobacteria	1B-05(2), 1B-08, 1B-38, 1B-39	2B-28	Not found
Bacteria	Actinobacteria	1B-01, 1B-11, 1B-12, 1B-15, 1B-16, 1B-17, 1B-20(2), 1B-23, 1B-34(2), 1B-35, 1B-42, 1B-43	2B-07, 2B-09(2), 2B-14(2), 2B-20, 2B-21, 2B-45(2), 2B-47	Not found
Bacteria	α -Proteobacteria	Not found	2B-26, 2B-34	Not found
Bacteria	Bacilli	1B-30	Not found	Not found
Bacteria	Chloroflexi	1B-18, 1B-22, 1B-28, 1B-31, 1B-32	2B-02, 2B-04(4), 2B-08(3), 2B-13, 2B-16, 2B-18, 2B-19, 2B-33(2), 2B-37, 2B-39, 2B-40	3B-06, 3B-27(4), 3B-44(12)
Bacteria	Clostridia	1B-21	2B-17	Not found
Bacteria	δ -Proteobacteria	Not found	2B-31	3B-12, 3B-17, 3B-26(6), 3B-43(3), 3B-45(2), 3B-48(4)
Bacteria	γ -Proteobacteria	1B-02, 1B-03, 1B-14(2)	2B-06, 2B-43, 2B-48	Not found
Bacteria	Nitrospira	1B-07, 1B-46	2B-30(2)	Not found
Bacteria	OP-11	1B-26, 1B-47	Not found	3B-14, 3B-28
Bacteria	Planctomycetacia	1B-09, 1B-25, 1B-29(2), 1B-40, 1B-48	2B-23	3B-02, 3B-08
Bacteria	Unclassified bacteria	1B-10, 1B-24(3), 1B-33, 1B-41(2), 1B-44	2B-05, 2B-32, 2B-36, 2B-12, 2B-25(2)	3B-09, 3B-22, 3B-37, 3B-18(2), 3B-19, 3B-21

Numbers between brackets represent the total number of sequences detected for each phylotype.

^aSequence names representative for unique phylotypes (<97% sequence similarity with known 16S rDNA sequences) are shown.

^bUnclassified archaea detected in deep-sea sediments.

*[#][†]Sequences belonging to the same phylotype as illustrated by similar underlining.

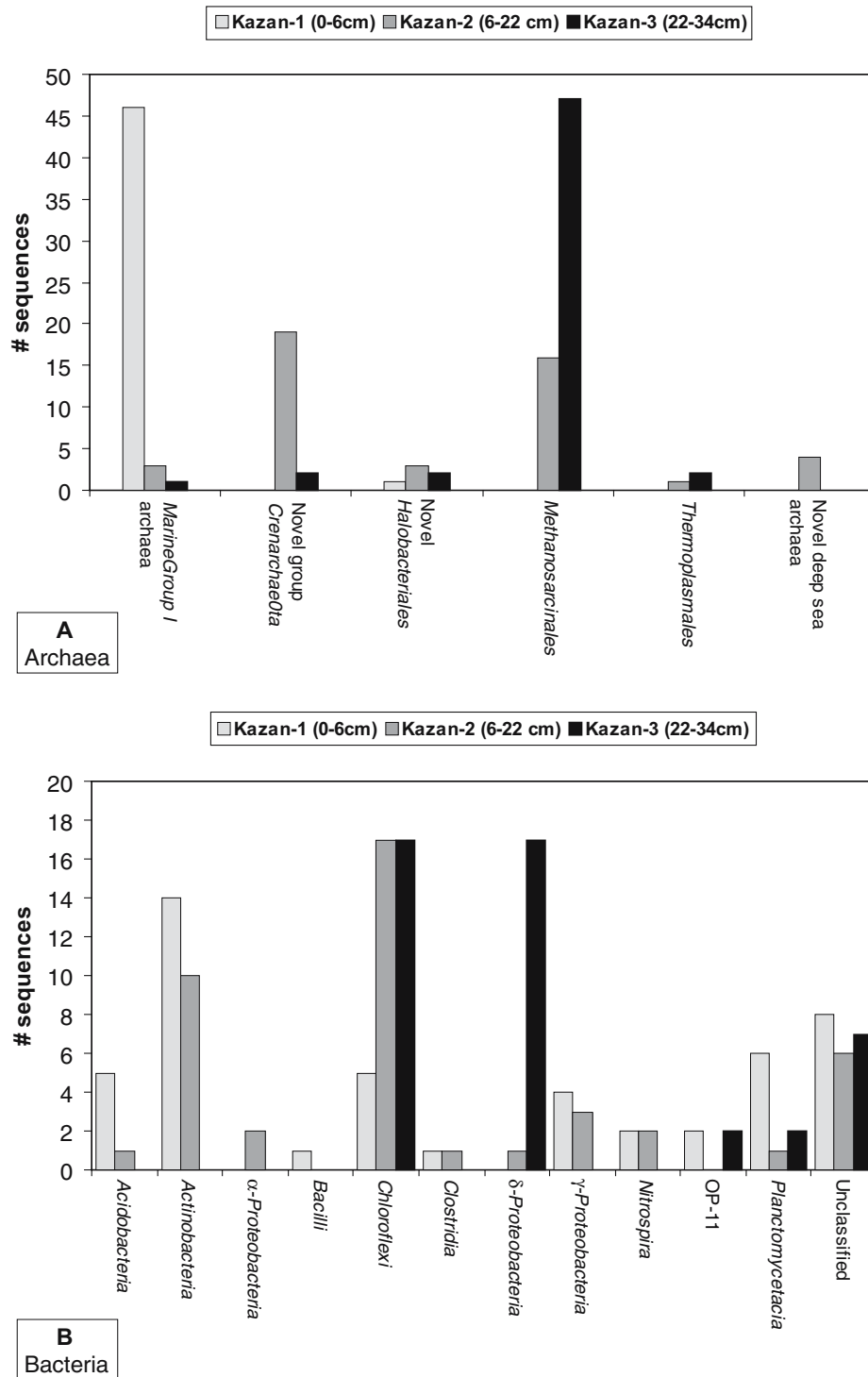


Figure 1. Distributions of archaeal (A) and bacterial (B) phylogenetic groups in three sediment depth layers collected from an active seep at Kazan mud volcano in the Eastern Mediterranean.

past/). The similarity between sediment layers at the level of phylotypes and phylogenetic groups was determined by using the Morisita–Horn index of similarity [56].

The 16S rRNA gene sequences determined in this study were deposited in Genbank under accession numbers AY591932–AY592229.

Results

Chemical Environment. Upon opening of the cores containing the Kazan mud volcano sediment samples, a strong sulfide smell was noticed. Furthermore, strong degassing was apparent, as deeper layers had a “mousse”-

Table 3. Morisita–Horn similarity matrix of bacterial and archaeal sequences at phylotype and phylogenetic group level in three depth layers of deep-sea sediment samples, collected from an active seep at Kazan mud volcano (Eastern Mediterranean)

	Kazan-1 (0–6 cm)						Kazan 2 (6–22 cm)						Kazan 3 (22–34 cm)					
	Bacteria			Archaea			Bacteria			Archaea			Bacteria			Archaea		
	Phylotype		Group	Phylotype		Group	Phylotype		Group	Phylotype		Group	Phylotype		Group	Phylotype		Group
	1	0.01	0	1	0.02	0	1	0.01	0.71	0.02	1	0.11	0	0.01	0.27	0	0.10	0.50
Kazan-1 (0–6 cm)	1			1			1			1			1			1		
Kazan-2 (6–22 cm)	0.01	1	0.71	0.02	1	0.11	0.01	1	0.71	0.10	1	0.11	0.01	0.64	0.27	0.10	0.50	1
Kazan-3 (22–34 cm)	0	0	0.27	0	0.02	0.02	0	0	0.64	0.10	0.50	0.50	1	1	1	1	1	1

like appearance. These observations indicated that the sediment was derived from an active methane seep, an observation that was supported by onboard methane measurements [54].

Analysis of the chemical parameters of the sediments (Table 1) showed the presence of nitrate in the top (Kazan-1) and middle (Kazan-2) layers. Sulfide was not detected in Kazan-1, but increased in concentration in the deeper layers, with the highest concentrations in Kazan-2. Sulfate concentrations were highest in the top layers, and, in gross terms, decreased with depth. Overall methane concentrations increased with depth; however, gas hydrate destabilization and degassing affected these measurements. Concentrations up to 60 mM methane were estimated to occur in the deepest sediment layer using model calculations [22]. TDOC values were roughly similar between the sediment layers studied.

Overall, the Kazan sediment thus showed clear stratification in chemical terms, with conditions propitious for anaerobic methane oxidation and sulfate reduction prevailing in the middle and deepest layers.

Molecular Community Analyses Based on Clone Libraries. The DNA extracted from the sediment samples showed yields of 13.2, 20.4, and 16.4 ng per g of Kazan-1, Kazan-2, and Kazan-3 sediment. The DNA was readily amplifiable by direct 16S ribosomal RNA gene PCR, indicating its purity. Clone libraries of bacterial and archaeal 16S rRNA genes were constructed for the Kazan-1, Kazan-2, and Kazan-3 layers. In total, 137 bacterial and 147 archaeal 16S rRNA gene sequences were thus obtained, encompassing approximately 45 bacterial and 50 archaeal sequences from each sediment layer. Rarefaction analysis of the distribution of bacterial and archaeal 16S rRNA genes in the clone libraries yielded asymptotic accumulation curves, indicating that the clone libraries represented abundant (dominant) members of the microbial communities. For the bacterial clone libraries, coverage values were 13% (Kazan-1), 24% (Kazan-2), and 55% (Kazan-3), and for the archaeal ones, 85% (Kazan-1), 54% (Kazan-2), and 76% (Kazan-3). This indicated a high total bacterial diversity that was only partially represented by the clone libraries constructed, *versus* a better-covered archaeal diversity.

Archaeal Diversity. Overall, the archaeal 16S rRNA gene sequences represented 38 phylotypes, which fell into six phylogenetic groups (Table 2, Fig. 1A). All six groups belonged to the *Euryarchaeota* and *Crenarchaeota*, the two major groups in the domain Archaea. Sediment layers Kazan-1 and Kazan-2 shared only one phylotype (affiliated with *Marine Group I* archaea), whereas Kazan-2 and Kazan-3 shared two phylotypes (see the sequences underlined in Table 2). Thus, substantial differences existed in the archaeal community structures between the

Table 4. Sequence statistics and diversity index of bacterial and archaeal sequences in three depth layers of deep-sea sediment samples, collected from an active seep at Kazan mud volcano (Eastern Mediterranean)

	Kazan-1 (0–6 cm)		Kazan-2 (6–22 cm)		Kazan-3 (22–34 cm)	
	Bacteria	Archaea	Bacteria	Archaea	Bacteria	Archaea
No. of sequences	48	47	44	46	45	55
Taxa ^a	40	8	33	20	19	13
SW diversity index	3.63	1.63	3.39	2.57	2.52	1.70

^aNumber of different taxa identified at phylotype level.
SW: Shannon–Weaver index.

three sediment layers under study (Table 2, Fig. 1A). In Kazan-1, sequences affiliated with the *Marine Group I*-related archaea were most abundant, whereas those of novel *Crenarchaea* and *Methanosarcinales*-related archaea were most abundant in Kazan-2. In Kazan-3, sequences of *Methanosarcinales*-related archaea were most frequent (see Fig. 1A).

Bacterial Diversity. Overall, the bacterial 16S rRNA gene sequences included 91 phylotypes that comprised 13 phylogenetic groups (Table 2, Fig. 1B). At the phylotype level, bacterial sequences sampled were unique per layer, as no shared phylotypes were observed between layers. At the level of phylogenetic groups, we found several overlaps between layers, as shown in Table 2. In particular, sequences reflecting the presence of *Planctomycetacia* were prevalent in all layers. The majority of bacterial sequences in Kazan-1 were classified as *Actinobacteria*, whereas *Chloroflexi*-like sequences were most abundant in Kazan-2 and those related to *Chloroflexi* and δ -*Proteobacteria* in Kazan-3.

Similarity and Diversity Indices. Morisita–Horn indices of similarity were calculated to estimate the similarities of the bacterial and archaeal communities between the sediment layers (Table 3). At the level of phylotypes, the similarities for both communities in the three sediment layers were found to be low, which indicates that each layer contained a microbial community of distinct structure. When the similarity values were calculated at the level of phylogenetic groups, these were found to be higher, i.e., up to 0.71 (Table 3). This indicated that, although each layer still showed a distinct community structure, there was a higher level of phylogenetic group “overlap” between the three layers. The highest similarities occurred between adjacent layers, whereas the nonadjacent layers Kazan-1 and Kazan-3 showed the lowest similarities.

Diversities in the microbial communities in the different sediment layers were assessed by using the Shannon diversity index. For the bacterial communities, the values obtained (Table 4) were high for all layers, showing a decrease from top (Kazan-1) to bottom (Kazan-3), from 3.6 to 2.5, respectively. The diversity

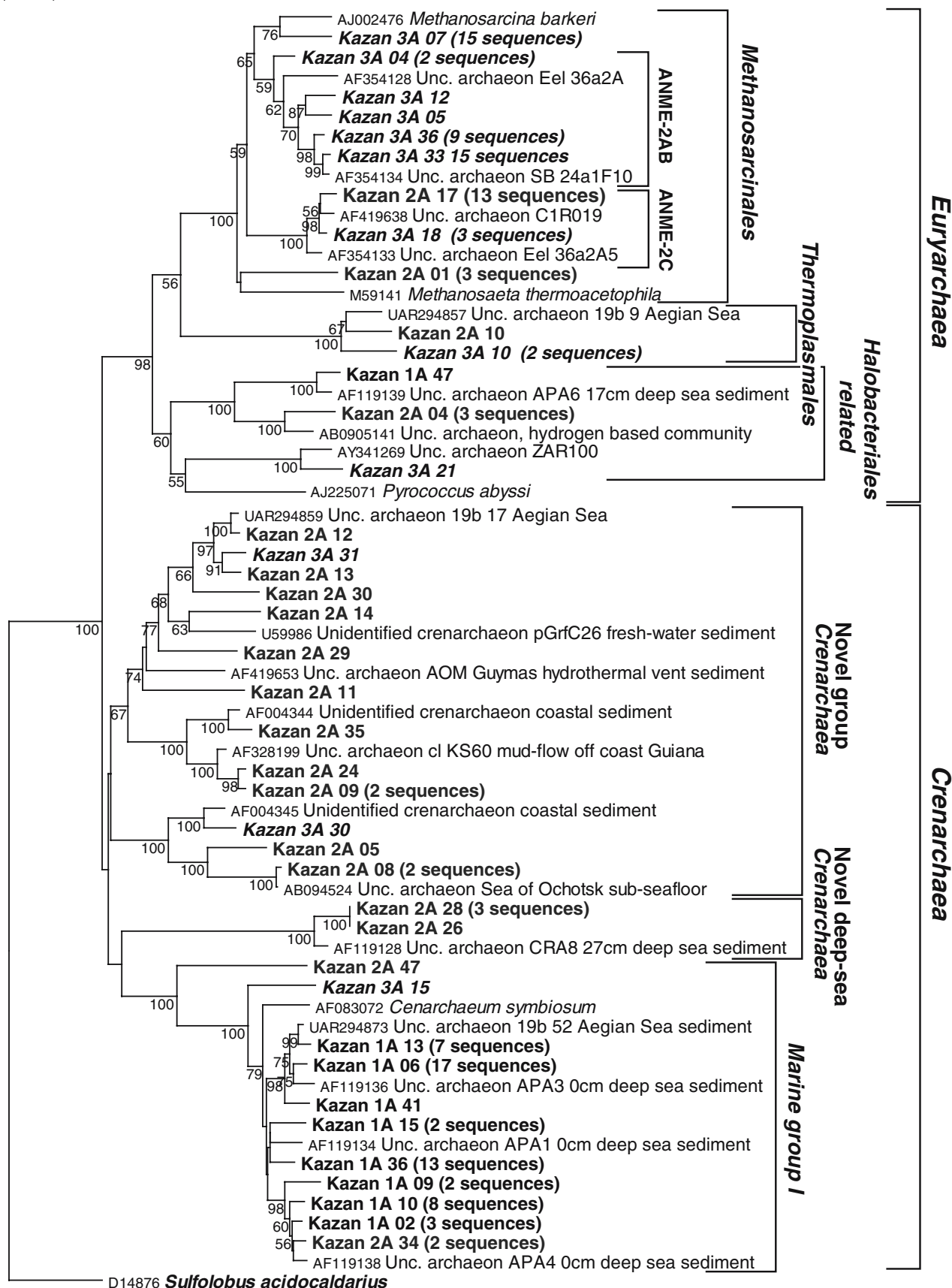
index for the archaeal communities ranged between 1.6 and 2.6 (Table 4), with low values in Kazan-1 and Kazan-3 and higher one in Kazan-2.

Grouping of Sequences and Link to Potential Function. Phylogenetic trees of the 16S rRNA gene sequences obtained in this study and those of the nearest relatives from Genbank were constructed to assess their phylogenetic relatedness (Figs. 2 and 3—only the most abundant bacterial sequences are included). Most archaeal and bacterial 16S rRNA gene sequences did not show high similarity (>97%) with sequences in the database (including sequences from cultivated species and environmental clones), indicating the likelihood that the main fraction of the prokaryotic community sampled was composed of novel organisms.

Kazan-1. The majority of the Kazan-1 archaeal sequences clustered within the *Marine Group I Crenarchaeota* (Figs. 1A and 2), which encompass heterotrophic as well as autotrophic organisms [14, 41, 57]. The other archaeal 16S rRNA sequences in Kazan-1 were related to sequences of the halophilic *Halobacteriales* (Figs. 1A and 2), cultured representatives of which include chemo-organotrophs capable of using amino acids or carbohydrates as carbon sources [38]. The bacterial sequences found in the Kazan-1 layer were related to *Acidobacteria*, *Actinobacteria*, and *Planctomycetacia* (Figs. 1B and 3), groups that include aerobic chemotrophs as well as aerobic and facultatively anaerobic heterotrophs [2, 18, 25]. In addition, sequences related to those of the γ -*Proteobacterial* sulfide-oxidizing bacteria such as *Beggiatoa* and *Thiobacillus* were present in this layer (Figs. 1B and 3).

Figure 2. Phylogenetic tree of archaeal 16S rRNA gene sequences in deep-sea sediment samples from three depth layers in sediment at the Kazan mud volcano, Eastern Mediterranean: Kazan-1 (0–6 cm), Kazan-2 (6–22 cm), and Kazan-3 (22–34 cm). The tree was constructed by using sequences longer than 800 bp, and neighbor-joining analysis using 1000 bootstrap replicates was used to infer the topology. Phylogenetic groups detected are indicated in brackets. The bar represents 5% sequence divergence.

0.05



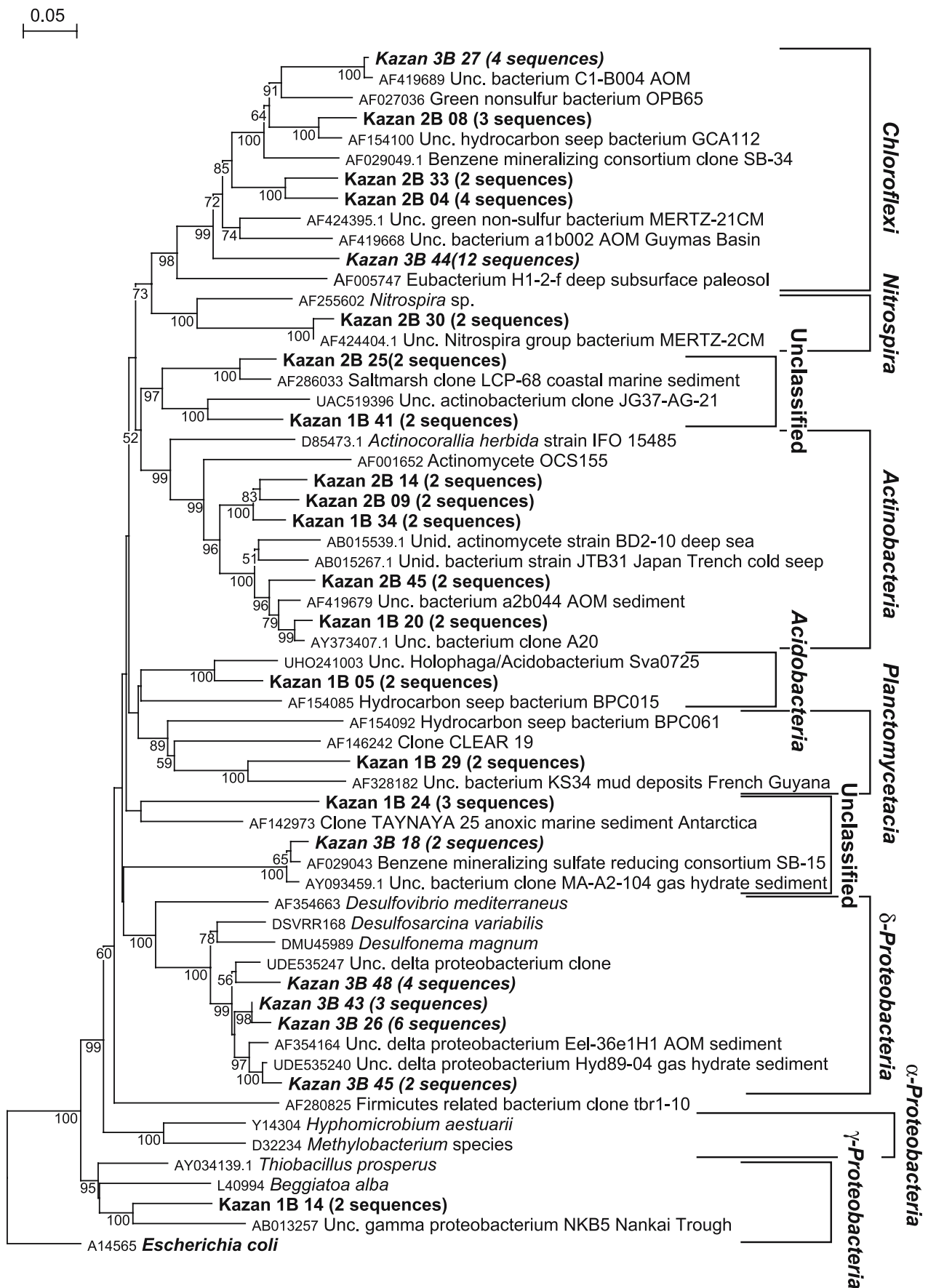


Table 5. Linking chemistry, lipid data, and 16S rRNA gene sequencing data to metabolic processes in three depth layers of deep-sea sediment samples, collected from an active seep at Kazan mud volcano (Eastern Mediterranean)

Metabolic process	Kazan-1 (0–6 cm)			Kazan-2 (6–22 cm)			Kazan-3 (22–34 cm)		
	16S ^a	Lipids ^b	Chemistry ^c	16S ^a	Lipids ^b	Chemistry ^c	16S ^a	Lipids ^b	Chemistry ^c
Anaerobic oxidation of methane (AOM)	–	±*	–	++	++	++	++	++	+
Anaerobic sulfate reduction	–	–	–	±	++	++	++	++	±
Aerobic sulfide oxidation	+	++	++	+	±	–	–	–	–
Heterotrophy (aerobic/anaerobic)	++	++	++	++	++	++	++	++	++

*–: No data indicating that this process occurred; ±: weak evidence indicating that this process occurred; +: strong evidence indicating that this process occurred; ++: conclusive data indicating that this process occurred.

^aFor 16S rDNA gene sequence data, –: no sequences; ±: 1–2 sequences; +: 3–10 sequences; ++: >10 sequences related to organisms with known or likely metabolic properties inferred from phylogenetic affiliation.

^bFor lipid data, –: lipids indicative for specific group of organisms not found; ±: lipids indicative for specific group found in low concentrations and/or carbon isotope values inconclusive; ++, lipids indicative for specific group found in high levels, with carbon isotope values supporting likely metabolic function (see Table 4).

^cFor chemistry data, AOM, –: no support from methane/sulfide and sulfate data; +: support from methane/sulfide and sulfate data (i.e., decrease of methane and increase of sulfide compared to adjacent layer, but sulfate data inconclusive); ++: strong support from methane/sulfide and sulfate data (i.e., decrease of methane and sulfate and increase of sulfide compared to adjacent layer). For *anaerobic sulfate reduction*, –: sulfate present in high levels, no sulfide detected; ±: sulfate present in high levels, but sulfide detected in high levels; ++: sulfate present in low levels and sulfide detected. For *aerobic sulfide oxidation*, –: sulfide present, no nitrate/nitrite detected, likely anaerobic conditions; ++: sulfide absent or sulfide gradient found previously (57), combined with the presence of nitrate/nitrite. For *heterotrophy*, ++: presence of organic matter, combined with the presence of ammonium (see Table 4).

Kazan-2. A large fraction (33%) of the archaeal sequences in Kazan-2 was related to methanogenic *Methanosarcinales* (Figs. 1A and 2). In fact, most of these sequences were closely related to those of the ANME-2 group, which has been implicated in AOM [23, 40]. Another large fraction of the archaeal sequences reflected the presence of *Crenarchaeota* with unknown metabolic properties (Figs. 1A and 2). Sequences of these *Crenarchaeota* have been previously obtained from deep-sea sediments [34, 49]. The majority of the bacterial sequences in Kazan-2 were related to those representative of *Chloroflexi* (Figs. 1B and 3), a metabolically versatile group of bacteria found in many environments [4]. In addition, the high frequency of the (heterotrophic) *Actinobacteria*-related sequences indicated that these bacteria were abundant in Kazan-2 (Figs. 1B and 3). Sequences affiliated with those of different α -*Proteobacteria*, notably the methylotrophic *Hyphomicrobium* and *Methyl-obacterium* species, were also detected, as were γ -*Proteobacteria* sequences that clustered with those of the sulfide oxidizers *Beggiatoa* and *Thiobacillus* (data not in tree). Hence, organisms involved in C₁ metabolism [12] and sulfide-based chemoautotrophy were indicated in Kazan-2; however, final proof of this concept should come from additional work using advanced methods such as activity-based (meta)genomics approaches. Interestingly,

the finding of a δ -*Proteobacteria*-related sequence, closely related to those of known sulfate-reducing bacteria (Fig. 1B, not in Fig. 3), indicated potential sulfate reduction.

Kazan-3. Most archaeal sequences in Kazan-3 were closely related to those of the ANME-2 group *Methanosarcinales* (Figs. 1A and 2). These sequences comprised at least three distinct groups, which clustered with the ANME-2 group related sequences of Kazan-2 (Fig. 2). As to the Kazan-3 bacterial community, 50% of the sequences clustered within those of the δ subclass of the *Proteobacteria* (Figs. 1B and 3), specifically with those of *Desulfobacteriaceae* (sulfate reducers) as well as other sequences from (1) environments propitious for AOM and (2) hydrocarbon-containing deep-sea sediments [29, 39]. The remaining bacterial sequences from Kazan-3 were related to those of *Chloroflexi*.

Overall, the chemistry and microbial community data indicated that the three sediment layers analyzed differed with respect to (1) the chemical environment, and (2) the relative dominance of specific types in the archaeal and bacterial populations, as a reflection of prevailing community processes.

Linking Chemical and Phylogeny Data to Lipid Marker Data. When the chemical, the lipid biomarker [54], microscopic observations [54] (A. Boetius, pers. comm.), and the phylogeny-based data indicative of function were compared (Table 5), great consistency regarding AOM and sulfate reduction was observed between the three datasets [22, 54]. In Kazan-1, the most abundant 16S rRNA gene sequences were related to those of—next to aerobic/facultatively anaerobic heterotrophs—autotrophs involved in sulfide or methane oxidation [14, 25, 41, 57]. The occurrence of these processes was supported by the

◀ **Figure 3.** Phylogenetic tree of the abundant bacterial 16S rRNA gene sequences in deep-sea sediment samples from three depth layers in sediment at the Kazan mud volcano, Eastern Mediterranean: Kazan-1 (0–6 cm), Kazan-2 (6–22 cm), and Kazan-3 (22–34 cm). The tree was constructed by using sequences longer than 800 bp, and neighbor-joining analysis using 1000 bootstrap replicates was used to infer the topology. Phylogenetic groups detected are indicated in brackets. The bar represents 5% sequence divergence.

chemistry and lipid data [54] (Tables 1 and 5). In Kazan-2, sequences reflecting (an)aerobic methane oxidizers, sulfide oxidizers, and sulfate reducers (1) were found, in addition to those of putative aerobes. The presence of such aerobes could indicate the occurrence of “bioirrigation,” thought to occur down to ~10 cm [54]. Bioirrigation might bring down O₂ within tubes and burrows into the anoxic zone and, therefore, aerobic prokaryotes may occur at the sulfidic/methanogenic and oxic interfaces. Evidence for sulfide- and methane-based chemotrophy was, to some extent, provided by the chemistry data, and the lipid biomarker data reported aerobic methanotrophs (or methylotrophs) and sulfide oxidizers, with carbon isotope compositions indicative of chemotrophic processes [44, 55]. Taken together, the microbial community in Kazan-2 likely contained organisms involved in anaerobic chemotrophy. The 16S rRNA gene sequences of Kazan-3 reflected the occurrence of microorganisms involved in AOM and/or sulfate reduction. The chemical data, mainly the methane and sulfate depth profiles, supported these processes [22, 54]. Strong support for their occurrence was also obtained from the carbon isotope values of the specific biomarker lipids [54]. In addition, evidence for the occurrence of organisms potentially involved in (anaerobic) heterotrophic processes below a depth of 10 cm was also obtained. The presence of anoxic conditions at this depth was supported by the presence of sulfide, whereas an increase in the concentration of ammonium with depth (Table 1) indicated the degradation of organic matter.

Discussion

What Do the Data Tell Us? Linking Chemical Environment to Phylogeny-Based Microbial Community Composition. We present a study based on directly obtained clone libraries of the microbial communities found at various sediment depths in the deep-sea cold seep Kazan. Much like other similar habitats [6, 23, 30, 36, 40, 51], the Kazan sediment can be characterized by counterposed gradients of several chemical compounds, under methane and sulfate, as drivers of microbial community structures and processes [54]. We refer to this publication for an extensive description of the dynamics of mud volcano chemistry. Specifically, the porewater analysis for the Kazan sediments showed clear changes in the concentrations of ammonia, nitrate, nitrite, sulfate, sulfide, and methane (Table 1), similar to those found in previous studies in marine sediments [46]. The pH values measured may have been affected by degassing upon sample retrieval; the differential values indicate higher CO₂ levels in the lower layers. We surmised that, in microbial terms, the sediment actually represented a large conglomerate of niches that are determined by the interactions of the varying gradients

over sediment length. Considering that all of the aforementioned chemical factors may drive the local microbial communities, it is difficult to ascertain which drivers are the most dominant. However, we hypothesized that the presence of the energy sources methane, sulfide, and (possibly) organic matter, in conjunction with that of the electron acceptors molecular oxygen, nitrate, and sulfate (and less oxidized forms of the latter two compounds), would be the primary drivers of the communities. Hence, we assessed (1) whether the microbial diversity per layer would actually report on the presence of such major driving forces, and (2) whether evidence for the presence of specific organisms potentially driven by a process such as AOM could be found. We will further bring forward a strategy to advance the phylogeny-based diversity *versus* function issue in this structured habitat.

The 16S rRNA gene sequence data generated in this study provided an overall picture of clear heterogeneity between the communities in the different sediment layers. One of the possible caveats in such 16S rRNA sequence-based studies is undersampling, i.e., the recovery and analysis of simply too few sequences to adequately describe the microbial community in its major facets. Recent statistical studies that addressed this issue for soil are actually ominous, in that unrealistically high numbers of sequences seem to be required to adequately sample a soil community [10, 11]. It is obvious that the habitat sampled by our team, deep-sea sediment, differs from soil in the extent and nature of its microbial diversity, which were actually unknown at the onset of this study. Hence, any prior estimation of the number of sequences required to obtain reasonable coverage of the total diversity could not be sensibly made. On the basis of the clone number analysed, the data seem to tell us that, grossly speaking, only dominant members of the microbial communities in the different sediment layers were sampled. On this basis, a picture of complex communities that are definitely unique per layer (although the full extent of diversity is as yet invisible from the data obtained) emerges. However, within these—still fragmentary—data, we can clearly discern the confines of the functional capabilities of such communities.

The main piece of evidence (Table 2)—i.e., the finding that sequences related to those of ANME-2-type archaea and to δ -proteobacterial sequences were abundant in Kazan-3 and to a lesser extent in Kazan-2—points in the direction of the AOM process taking place in these layers. This supports the conclusion that the local microbial communities dominated by ANME-2-type archaea and δ -proteobacterial sulfate reducers related to *Desulfosarcina* sp. are, to a large extent, driven by AOM. We tentatively link the lack of evidence for the presence of these organisms in Kazan-1 to them being less dominant in the light of the strong dominance of heterotrophs in this

layer. This was consistent with the absence of sulfide from this layer. Other studies also indicated that the ANME-2 group archaea, next to those of ANME-1, are prominent parts of communities involved in AOM [6, 23, 40, 51]. On the other hand, the absence from, or low abundance of ANME-1 in, our samples was indicated by the absence of characteristic membrane lipids [53, 55]. A recent comparison between AOM communities from different “methane-rich” deep-sea areas also showed differences in dominance of ANME1 or ANME2 [30]. These areas showed chemical conditions favorable for AOM (i.e., sulfate and methane concentrations of up to 2 and 60 mM, respectively in the zone of AOM) and a relatively low diversity in archaeal ANME phylotypes, which is consistent with our findings. ANME-2-type archaea were also abundant in Kazan-2, and evidence for the occurrence of *δ-Proteobacteria* capable of sulfate reduction was also found, albeit at low abundance. Direct microscopical observation of the sediment material revealed the presence of microbial aggregates that resemble those implicated in AOM [6] in these sediments (not shown, shown in an earlier work [54]).

The depth profiles of sulfate, sulfide, methane, and dissolved inorganic carbon (DIC) reflect the environmental conditions at the time of sampling, whereas by nature the microbial community data based on 16S rRNA gene sequences provide an integrated view of historical events [54]. Moreover, the data on salinity should also be interpreted in a similar manner. One could speculate that the differences observed between Kazan-1 and Kazan-2 on one hand, and Kazan-3 on the other, which might be indicative of recent gas hydrate dissolution, might have selected for specific microbial communities. However, direct evidence for this contention is lacking in this study. The sediment samples used in this study integrate depth intervals of 6, 16, and 12 cm, whereas the porewater chemical data had a resolution of 1 cm. As a consequence, the microbial communities as determined by the DNA analysis provide a more integrated view (and therefore less specific per smaller unit volume) than the porewater data.

Support from Lipid Biomarker Data. Lipid biomarker data were used as reference data that reflect functionally important microorganisms [54]. The summed concentrations of archaeol *sn*-3-hydroxyarchaeol and five unsaturated (PMI:x) compounds were used to indicate AOM archaea [53, 54]. Nonisoprenoidal dialkylethers were used as specific indicators of sulfate-reducing bacteria [42]. Isotopically depleted diploptene/diplopterol and bishomohopanol were used as lipids indicating aerobic methanotrophs or methylotrophs [16, 44] and aerobic sulfide-oxidizing prokaryotes [43, 54], respectively. These lipids were set in relation to the amount of total lipids extracted from the sediment layers

studied to illustrate the relative importance of the organisms presumably involved in the metabolic processes identified.

The abundance of characteristic bacterial and archaeal membrane lipids in the top 30 cm of the sediments of the Kazan mud volcano are presented in Table 1. Lipids assigned to archaea involved in AOM (i.e., archaeal, hydroxyarchaeol, and PMI:x) showed a strong increase in abundance with depth. A maximum abundance of this group was observed in Kazan-2. Previous studies showed strongly depleted $\delta^{13}\text{C}$ carbon isotopic values of these compounds, which were consistent with values for methane-derived carbon in AOM environments [54]. Lipids presumed to derive from aerobic methane oxidizing (or methylotrophic) bacteria were found in layers Kazan-1 and Kazan-2, with decreasing relative abundance downcore (Table 4). β,β -Bishomohopanol, tentatively attributed to sulfide-oxidizing bacteria [55], was most abundant in the top layer Kazan-1, but it was also present in layer Kazan-2. The carbon isotopic values of these compounds showed $\delta^{13}\text{C}$ values between -46‰ and -53‰ , which is consistent with values for chemotrophic processes [54]. Most characteristic bacterial lipids identified in the three sediment layers were indicative for sulfate-reducing bacteria (e.g., dialkyl ethers). These compounds were detected in the lowest sediment layers, be it that they were most abundant in Kazan-2. We are puzzled by the merely partial support for these organisms from the phylogeny-based data (Table 2); however, it is known that PCR-based detection of specific sequences from natural samples can be hampered. Nevertheless, their occurrence is likely, as previously reported carbon isotope measurements from the same sediment showed strongly negative $\delta^{13}\text{C}$ values of DIC and lipids derived from AOM archaea and sulfate-reducing bacteria [22, 54].

Linking Phylogeny to Function—Caveats and Approaches.

The high diversities and low similarities of the phylotypes in the microbial communities between the three Kazan sediment layers may reflect a range of different metabolic processes taking place in these layers resulting from, as well as yielding, different habitat chemistries. However, this study, like virtually all other studies on microbial communities, only assessed a subset of the total microorganisms present. Furthermore, it has solely relied on the commonly used phylogenetic marker, the 16S ribosomal RNA gene sequence, to unravel the microbial communities. Use of this marker to indicate function assumes that functional properties are conserved among phylogenetically related populations, and that the function of “novel” organisms can be inferred by comparison with species that have been previously cultivated and characterized [21, 50]. It is known that there are pitfalls in these assumptions, and a cautious approach is

therefore indicated [9, 21, 26, 45, 50]. For instance, organisms occurring in the different niches in the sediment layers may have diversified at either the 16S rRNA gene or the functional gene levels, processes which both disentangle the link between phylogeny and function [17]. In addition, lateral gene transfer among prokaryotes is a significant force in microbial evolution, resulting in metabolic functions that show a paraphyletic distribution [48]. This has created uncertainty about the interpretation of taxonomies based on gene sequences in relation to phenetic taxonomy [17, 48].

One way to resolve the apparent riddle posed by the large diversity of phylogenetic types in the different sediment layers and the difficulty of firmly linking such types to function would obviously be to take a metagenomics approach that is possibly coupled to either stable isotope probing or DNA-based pre-enrichment methodology. These strategies strongly depend on the production of sufficient quantities of highly pure microbial community DNA clonable in, e.g., fosmid vectors. The 16S rRNA-based sequences found to be most dominant in this study, e.g., the ANME-2 related sequences, might be useful as probes that can either “fish out” the underlying organisms from a sediment DNA pool or detect these at the cell level. Once DNA has been obtained (enriched), one might apply metagenomic cloning and sequencing to directly investigate the genetic link between phylogeny and potential function (on the basis of the sequences of the genes involved) and to ascertain whether the underlying organisms are indeed functionally dominant. The genetic information thus unlocked may then yield suitable probes or primer systems applicable in mRNA-based measurements.

Using such approaches, a direct link can theoretically be made between the presence and the activities of the populations that play key roles in deep-sea sediments. To accomplish this and at the theoretical level, the identities and activities of the numerically dominant microorganisms in cold seeps should be further examined with respect to their importance in the consumption of methane or sulfide. An emphasis should be placed on the *in situ* detection of numerically abundant populations and their respective activities, for instance, via experiments in which the metabolism of labeled substrates (either ^{13}C - or ^{14}C -labeled) is combined with fluorescent *in situ* hybridization combined with stable carbon isotope measurement (FISH-SIMS) or substrate-tracking autoradiography fluorescent *in situ* hybridization (STAR-FISH). These activity measurements could be combined with molecular detection methods such as “real-time” reverse transcription-PCR (RT-PCR) with sediment 16S rRNA as a template, DNA microarrays for functional gene analysis, and FISH to identify the abundance, activity, and viability of microbial populations.

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